

Stimulation of an $\alpha 1$ -adrenergic receptor downregulates ecto-5' nucleotidase activity on the apical membrane of RPE cells

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Abstract

The purines ATP and adenosine play an important role in the communication between the photoreceptors and the retinal pigment epithelium (RPE). While the RPE is known to release ATP into subretinal space, the source of extracellular adenosine is unclear. In other tissues, ecto-nucleotidases mediate the consecutive dephosphorylation of ATP to AMP, and AMP is converted to adenosine by ecto-5' nucleotidase (CD73). This study identifies ecto-5' nucleotidase on RPE cells and investigates modulation of enzyme activity. The RPE was the most active site of 5'AMP dephosphorylation in the posterior rat eye. The ecto-5' nucleotidase inhibitor $\alpha\beta$ mADP prevented the production adenosine by the apical membrane of the bovine RPE. Cultured human ARPE-19 cells expressed mRNA and protein for ecto-5' nucleotidase. The production of phosphate from 5'AMP by ARPE-19 cells was inhibited by $\alpha\beta$ mADP, but the ecto-alkaline phosphatase inhibitor levamisole had no effect. Degradation of 5'AMP was blocked by norepinephrine, epinephrine and phenylephrine, with inhibition by antagonists prazosin and corynanthine implicating the $\alpha 1$ adrenergic receptor. The block of enzyme activity by norepinephrine was rapid, occurring within 1 min, and was similar at both 4 and 37°C, consistent with cleavage of the enzyme from its GPI anchor. HPLC measurements indicated norepinephrine reduced levels of adenosine in the bath. In the apical face of the bovine-RPE eyecup, norepinephrine reduced the production of phosphate from 5'AMP, suggesting that both receptor and enzyme face sub-retinal space. In conclusion, RPE cells express ecto-5' nucleotidase, with activity on the apical membrane, and stimulation of $\alpha 1$ adrenergic receptors downregulates activity. As epinephrine is released at light onset, and adenosine can inhibit phagocytosis, the corresponding decrease in subretinal adenosine levels may contribute to the enhanced the phagocytosis of rod outer segments that occurs at this time.

Introduction

The retinal pigment epithelium (RPE) is in close physical alignment with the outer segments of the retinal photoreceptors and performs a variety of roles to maintain the optimal photoreceptor function. The complex interaction between the two cell types requires a tightly-regulated neurochemical communication, to which the purines ATP and adenosine contribute. The apical membrane of the RPE, which faces sub-retinal space, contains a P2Y₂ receptor for ATP [1]. Stimulation of this receptor enhances movement of fluid across the tissue and into the choroidal circulation [2], and agonists for this receptor can reduce retinal detachments by increasing the rate of fluid

reabsorption from the subretinal space [3]. Stimulation of an A₂ receptor for adenosine can decrease phagocytosis of rod outer segments by the RPE [4]. This phagocytosis is an essential component of photoreceptor outer segment turnover which ensures the optimal photoreceptor function. The endogenous source and regulation of subretinal purines could consequently impact both fluid movement and phagocytosis.

ATP is known to be released across the apical membrane of the RPE into subretinal space [5, 6] and recent evidence indicates the cystic fibrosis transmembrane conductance regulator (CFTR) is involved in this release under at least some circumstances [7]. However, the source of extracellular adenosine is currently unclear. In some tissues, extracellular adenosine arises from the actions of an adenosine transporter [8], but in many epithelial cells the majority of adenosine is produced by the consecutive dephosphorylation of ATP to ADP, AMP and adenosine by a series of extracellular enzymes [9, 10]. ATP can be

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converted into AMP and pyrophosphate by members of the ectonucleotide pyrophosphatase/phosphodiesterase (eNPP) family, while ecto-nucleoside triphosphate diphosphohydrolase (NTPDases) can mediate either the single dephosphorylation of ATP into ADP (NTPDase 2) or the double dephosphorylation of ATP into AMP (NTPDases 1 and 3) [11, 12]. Multiple members of both the NTPDase and eNPP families have been identified in RPE cells [13].

The dephosphorylation of AMP into adenosine by the RPE is less well understood. A previous electron microscopy study demonstrated that enzyme activity on the apical membrane of rat RPE could produce phosphate from exogenous 5'AMP [14]. However the molecular identification of the enzyme, its physiologic impact on subretinal adenosine levels and its regulation remain unknown. The present study examines ecto-5' nucleotidase activity on the apical membrane of RPE cells and how the activity of this enzyme can be modified. In the first part, ecto-5-nucleotidase is identified on fresh and cultured RPE cells using pharmacological, molecular and histochemical approaches. The second part indicates enzyme activity can be modified by catecholamines acting at $\alpha 1$ adrenergic receptors on the apical membrane. As epinephrine may be released by the retina following illumination [15], the production of adenosine by ecto-5' nucleotidase may provide a functional link between light and RPE function.

Materials and methods

All reagents were obtained from Sigma Chemical Corp. (St. Louis, MO) unless otherwise indicated.

Cell culture

The human ARPE-19 cell line [16] was obtained from the American Type Culture Collection (Manassas, VA) and grown in a 1:1 mixture of Dulbecco's modified Eagles medium (DMEM) and Ham's F12 medium with 3 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin and 2.5 mg/ml Fungizone (all Invitrogen Corp, Carlsbad, CA) and 10% fetal bovine serum (FBS, HyClone Laboratories, Inc., Logan, UT). Cells were incubated at 37°C in 5% CO₂ and subcultured with 0.25% trypsin and 0.02% EDTA.

Bovine RPE eyecup preparation

Bovine eyes were bisected at the ora serrata and the retina removed as described previously [7, 13]. This bovine RPE eyecup preparation has the apical membrane facing the cup interior, with samples from the cup consequently reflecting activity on the apical membrane. After rinsing the eyecup, 5'AMP or ATP was added in a total volume of 1 ml. For adenosine measurements, isotonic solution composed of (in mM) 105 NaCl; 5 KCl; 6 HEPES Acid; 4 NaHEPES; 5 NaHCO₃; 60 Mannitol; 5 Glucose; 0.5 MgCl₂; 1.3 CaCl₂, at pH 7.4. For phosphate measurements, a glucose-free

0.1 mM TRIS HCl at pH 7.5. Norepinephrine was included in some preparations. After 20 min, a 600 μ l sample was removed and frozen at -20°C. Samples were assayed for adenosine or phosphate as indicated.

Adenosine measurements

High Performance Liquid Chromatography (HPLC) analysis was performed as previously published [13]. To control for biological and experimental variability between samples obtained on different days, the amount of adenosine was normalized to control levels for each trial.

Lead precipitate assay

Activity of 5' nucleotidase was assayed based upon method of Schoen and Kreutzberg [17] in which inorganic phosphate released in the conversion of 5'AMP to adenosine reacts with lead nitrate to produce a lead phosphate precipitate. Adult albino Sprague-Dawley rats were anesthetized with intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine followed by a larger lethal dose, then perfused with 4% paraformaldehyde. All treatment of animals conformed to the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. Enucleated eyes were usually postfixed with 4% paraformaldehyde for 3 h, followed by immersion in 30% sucrose before embedding in OCT and rapid freezing. Cryostat sections were cut at 14 μ m and briefly washed with 4% paraformaldehyde before beginning the 5'-nucleotidase assay. Sections were incubated for 30 min at 37°C in 80 mM Tris malate buffer, 0.12% lead nitrate, 10 mM MgSO₄ and either 50 mg/ml 5'AMP or 50 mg/ml 2'AMP (137 mM). After washing, the reaction was developed by incubating the tissue sections in 20% ammonium sulfide for 5 min at room temperature. Slides were washed and visualized with an upright microscope (Nikon Eclipse 600, Nikon) and digital images obtained with a 3-CCD digital camera (Toshiba America, Irvine, CA) and Image Pro Plus software (Media Cybernetics, Silver Spring, MD). While similar amounts of lead precipitate were formed on fixed and unfixed sections, the retinal structure was better maintained with the fixed tissue.

Immunohistochemistry

Retinal sections from adult albino rats were processed as described above. After washing in PBS, tissue was incubated with mouse anti-CD73 antibody diluted 1:1,000 (Clone Ty-73, BD Pharmingen, San Jose, CA) in PBS with 10% Superblock (Pierce Biotechnology Inc. Rockford, IL.) in 0.1% Triton X-100 and 0.1% Tween-20 for 4 h at 25°C. After wash, tissues were incubated with biotinylated donkey anti-mouse (Jackson ImmunoResearch, West Grove, PA, 1:500 in PBS) for 2 h at 25°C, followed by Streptavidin-conjugated Cy3 fluorophore for 1 h at 25°C. A parallel incubation with IgG (1:500, Jackson ImmunoResearch) acted as a negative control. After washing, cells were mounted with a fluorescent mounting medium

containing DAPI to visualize the nuclei (H-1200, Vector Labs, Burlingame, CA). Images were taken on a Nikon Eclipse 600 upright microscope equipped for epifluorescence with a 3-CCD digital camera and analyzed on line using Image Pro Plus software: DAPI was imaged with 360 nm excitation and >515 nm emission, while Cy3 was excited at 540 nm with emission >590 nm.

ARPE-19 cells were grown on Lab-Tek-II Chamber slides (Nalgene Nunc Int. Corp., Naperville IL) overnight using culture medium described above. Cells were fixed using warm 4% paraformaldehyde added to the medium in a 1:1 ratio for 2 min, then straight warm 4% paraformaldehyde for 30 min. Cells were washed 3 × 5 min in PBS, after which cells were incubated for 18 h at 4°C with a goat polyclonal antibody to ecto-5' nucleotidase CD73 (# SC-14684, Santa Cruz Biotechnology, Santa Cruz, CA; 1:250 in PBS). Cells were incubated with biotinylated anti-goat antibody (Jackson ImmunoResearch, 1:1,000 in PBS) for 2 h at 25°C, washed and incubated in streptavidin-linked Cy3 (Jackson ImmunoResearch, 1:1,000 in PBS) for 1 h at 25°C. To ensure specificity of the antibody binding, two controls were performed. Omission of the primary antibody eliminated all staining, while pre-incubating with CD73 peptide (Santa Cruz Biotechnology), eliminated >80% of the staining (data not shown).

RT-PCR

RNA was extracted from ARPE-19 cells using the Trizol reagent (Invitrogen, Carlsbad, CA) RNA was treated with RQ1 RNase-free DNase I (Promega, Madison, WI.) Reverse transcription was performed from 1 µg of total RNA using the SuperScript First Strand Synthesis system (Invitrogen, Carlsbad, CA). Primers were obtained from a previously published paper [18]: Sense: 5'-CAC-CAAGGTTTCAGCAGATCCGC-3', Antisense: 5'-GTTTCATCAATGGGCGACCGG-3', 1,006 basepair product. PCR was performed with 2 µl SuperScript product, 2 mM MgCl₂ and 0.4 nM of each primer, using 0.5 µl AmpliTaq Gold Polymerase system (Applied Biosystems, Foster City, CA.) for 1 min 95°C, 1 min 55°C, 2 min 72°C, 35 cycles, with a final extension step of 20 min 72°C. SuperScript was omitted from the negative control. The band was visualized on a 2% agarose gel, photographed, and purified from a 2% LMP agarose gel using the QIAEX II Gel Extraction kit (QIAGEN, Valencia, CA.) Cloning was performed using a TOPO TA cloning kit with Top 10 F' cells (Invitrogen, Carlsbad, CA.) and purified using the Wizard Plus Minipreps kit (Promega, Madison, WI.) The DNA was sequenced at the University of Pennsylvania Cell Center Sequencing Facility. The resulting sequence was identified using BLAST nucleotide database (<http://www.ncbi.nlm.nih.gov>).

Measurement of phosphate production

Phosphate production was determined using the P_iPer Phosphate Assay Kit in basic accordance with the manufacturer's instructions (Molecular Probes/Invitrogen,

Eugene, OR). A standard curve constructed with 0 to 400 µM Pi from 20 µM increments indicated that absorbance at 565 nm provided an accurate measure of Pi present in the bath. ARPE-19 cells were grown to confluence in 24 well dishes, washed twice with 0.1 mM TRIS HCl pH 7.5, and incubated with working solution (control), or solution including AMP with or without drugs. All experiments were performed for 30 min at 37°C according to the manufacturer's protocol except where indicated in Figure 5. α1-adrenergic antagonists were present 30 min before and during the reaction. To test the effect of temperature, all solutions and plates were precooled to 4°C for 30 min before the experiment began, with reaction itself also occurring at the appropriate temperature. After all incubations, 0.3 ml was drawn from each well for analysis. Absorbance of each sample was read at 565 nm using a spectrophotometer (Milton Roy, Ivyland, PA). In experiments involving levamisole, the phosphate signal was assessed on cells grown in 96 well plates with a Fluroskan Ascent fluorimeter (ThermoElectron, Milford, MA). All drugs were tested on the assay in the absence of cells and found to have no effect.

Data analysis and materials

Data are expressed as means ± SEM. Significance was evaluated using a one-way ANOVA with Tukey post test where more than two variables were present or an unpaired Student's *t* test when only two variables were present. Significance was defined as *p* < 0.05.

Phosphate production was quantified by normalizing data to the mean value for control records (5'AMP alone). The percent (%) block of enzyme activity was defined as:

$$100 \cdot (a - x) / (a - c),$$

where *a* is the mean absorbance at 565 nm in the presence of 5'AMP, *c* is the mean absorbance in the absence of 5'AMP and *x* is each absorbance in the presence of 5'AMP + blocking compound after correction for background levels without added 5'AMP. Phenylephrine concentration-response curve was fit with an exponential decay function

$$f = y_0 + a \cdot (\exp^{-bx})$$

using the least squares method of Sigmaplot software (SPSS Inc, Chicago, IL) after subtracting control values before normalization to the mean level of 5'AMP alone of each day.

Results

Identification of ecto-5' nucleotidase on fresh and cultured RPE cells

Initial experiments were performed to determine if RPE cells dephosphorylated AMP to adenosine and to identify the enzyme responsible for this conversion. First, a lead

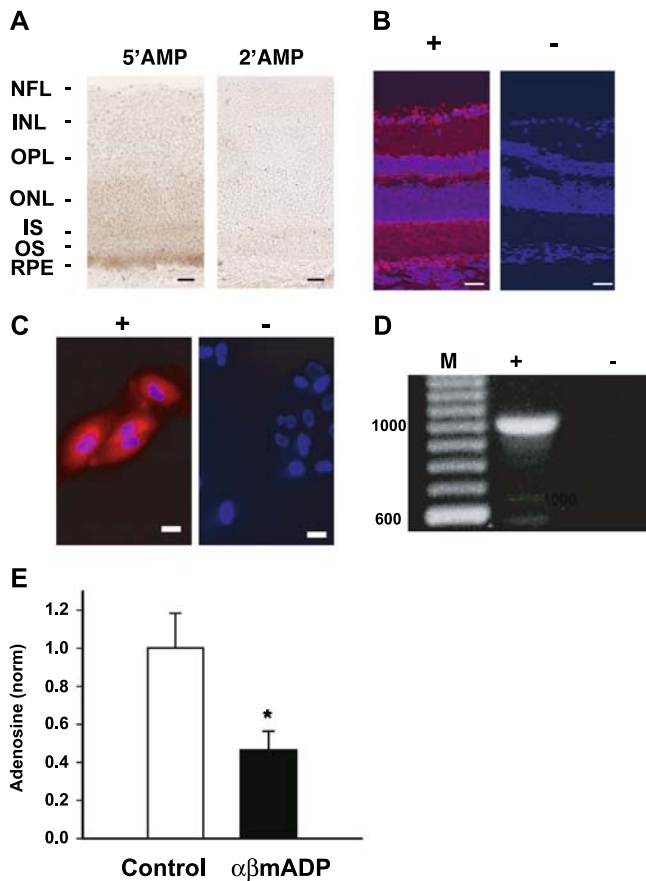


Figure 1. Identification of ecto-5' nucleotidase on the RPE. (A) Sections of albino rat retina incubated with 5'AMP show an intense lead phosphate precipitate at the level of the RPE, indicating 5' nucleotidase activity is concentrated there (*left panel*). No reaction product was detected when 2'AMP was used as a substrate (*right panel*). NFL nerve fiber layer, INL inner nuclear layer, OPL outer plexiform layer, ONL outer nuclear layer, IS inner segments, OS outer segments, RPE retinal pigment epithelium. Scale bars = 25 μ M. (B) Immunohistochemical staining localizes the ecto-5' nucleotidase antigen in RPE layer in *panel '+'*; In *panel '-'*, IgG was used instead of the specific antibody as a control. Blue indicates nuclei stained with DAPI and confirms the identification of retinal layers. Scale bars = 50 μ M. (C) Ecto-5' nucleotidase antigen is also present on cultured human ARPE-19 cells. *Panel '+'* indicates positive control while *panel '-'* indicates absence of primary antibody. Scale bars = 25 μ M. (D) RT-PCR reaction with ecto-5' nucleotidase (CD73) primers produces band of predicted 1,006 bp size on RNA obtained from ARPE-19 cells in *lane '+'*. No band was seen when reverse transcriptase was omitted from the reaction (*lane '-'*). Lane 'M' shows size markers. (E) The production of adenosine by the apical membrane of the fresh bovine RPE eyecup from ATP was inhibited by 100 μ M ecto-5' nucleotidase inhibitor $\alpha\beta$ mADP. Levels were normalized to the mean control values of area under the HPLC curve for each experimental set. * $p = 0.018$, $n = 8-9$.

precipitate assay was performed on sections from the albino rat retina. Excess 5'AMP was used as a substrate to determine regions of ecto-5' nucleotidase activity. Addition of 5'AMP to the reaction resulted in intense precipitate banding at the level of the RPE/outer segments (Figure 1A). Activity was considerably higher in the RPE layer than in any other tissue of the retina, although lighter precipitate was also detected at the level of the inner segments. The use of 2'AMP as a substrate, instead of 5'AMP, eliminated any reaction product.

The presence of ecto-5' nucleotidase in the RPE was confirmed immunohistochemically. Bright staining was detected in the RPE layer, with more diffuse localization throughout the retina including the nerve fiber layer (Figure 1B). Counter staining of cell nuclei with DAPI allowed identification of retinal layers and emphasized that these layers were normal, displaying no sign of damage that can affect albino animals. Cultured human ARPE-19 cells also stained positively for the enzyme (Figure 1C), while RT-PCR on RNA from ARPE-19 cells confirmed the identification of ecto-5' nucleotidase (CD73) on a molecular level. Primers for CD73 produced an intense band of predicted 1,006 base pairs (Figure 1D). The sequenced product showed >99% similarity with published CD73 sequences (accession # NM_002526, corresponding to ecto-5'-nucleotidase).

The polarity of enzyme activity was further examined using the bovine RPE-eyecup. In this preparation, the apical membrane of the eyecup is in contact with the solution. As the tight junctions of the RPE monolayers were intact, this likely reflects activity on the apical membrane [7]. The ability of extracellular enzymes to convert ATP to adenosine was measured directly by adding 100 nM ATP to the inside of the bovine RPE eyecup and measuring the adenosine levels with HPLC. The contribution of ecto-5' nucleotidase to this conversion was determined with the compound $\alpha\beta$ mADP, an effective inhibitor of 5' nucleotidase at physiologic pH [19]. Extracellular adenosine levels were reduced >50% by incubation with 100 μ M $\alpha\beta$ mADP (Figure 1E). This is consistent with conversion of the 5'AMP to adenosine by ecto-5' nucleotidase on the apical membrane of bovine RPE cells.

Production of phosphate

Phosphate production was measured with the P_iPer phosphate assay in the presence of excess 5'AMP. Baseline phosphate levels were 30.8 ± 1.4 μ M and rose to 99.1 ± 6.6 μ M ($n = 7$) after a standard 30 min incubation with 100 μ M 5'AMP, although the relative rise did vary somewhat in different trials. As phosphate can be produced by extracellular enzymes in addition to ecto-5' nucleotidase, several controls were performed to ensure specificity of the reaction. Phosphate levels remained near baseline when cells were incubated with 2'AMP, suggesting this nucleotide was a negligible source of phosphate (Figure 2A). The production of phosphate from 5'AMP was also eliminated by 100 μ M $\alpha\beta$ mADP (Figure 2B). However, the alkaline phosphatase inhibitor levamisole (10 mM) had no effect on phosphate production (Figure 2C). These results are consistent with activity of ecto-5' nucleotidase, and support the predominant role of ecto-5' nucleotidase in the production of phosphate from 5'AMP in these cells.

Regulation of ARPE-19 cells ecto-5' nucleotidase activity by norepinephrine

Indirect evidence from studies on manganese-dependent pyrimidine 5' nucleotidase suggests enzyme activity in the

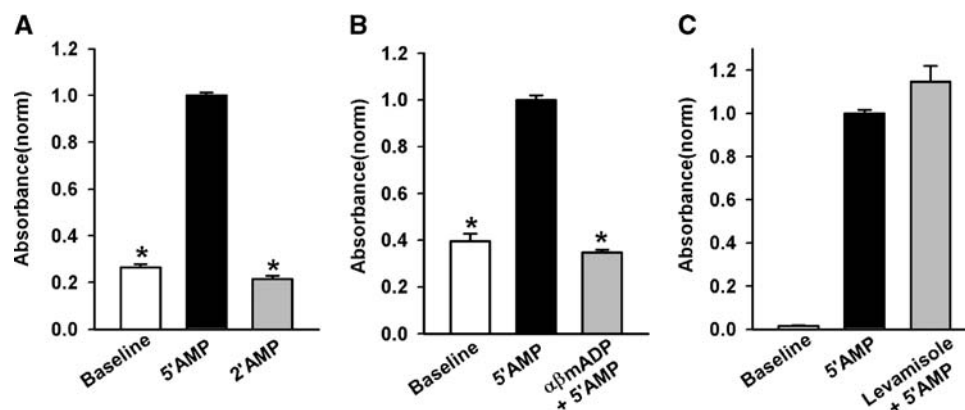


Figure 2. Verification of phosphate assay measured ecto-5' nucleotidase activity. (A) Intact ARPE-19 cells produce considerably more Pi, as detected by absorbance of resorufin, when bathed with 5'AMP than 2'AMP when both nucleotides were used at 100 μ M. Here and throughout the study, data are normalized to the mean absorbance value for 5'AMP for each trial. Baseline represents background levels without added substrate. All compounds had no effect on the assay itself. * p < 0.05 compared to 5'AMP, 2'AMP vs. control not significant (NS), n = 4. (B) The production of Pi by intact ARPE-19 cells is completely blocked by the ecto-5' nucleotidase inhibitor $\alpha\beta$ mADP (100 μ M). Although cells from both 5'AMP and $\alpha\beta$ mADP populations were bathed in 100 μ M 5'AMP, the inhibitor prevented the production of Pi. * p < 0.05 vs. 5'AMP, $\alpha\beta$ mADP vs. control NS, n = 3. (C) The alkaline phosphatase inhibitor levamisole (10 mM) had no effect on phosphate production in the presence of 100 μ M 5'AMP. * p < 0.05 vs. 5'AMP, n = 12–20.

RPE may be decreased upon onset of light [20]. Numerous changes accompany the transition from dark to light, including the release of epinephrine from the retina at light onset [15]. We thus asked whether catecholamines could reduce the activity of the ecto-5' nucleotidase present on the RPE.

Addition of 10 μ M norepinephrine to the assay mixture bathing ARPE-19 cells reduced the extracellular production of phosphate by 51%, corresponding to a decrease of

80% when baseline Pi levels in the absence of added 5'AMP were subtracted (n = 10–13, Figure 3A). Additional experiments were performed to verify the ability of norepinephrine to decrease ecto-5' nucleotidase activity. ATP was added to the solution bathing the ARPE-19 cells and levels of adenosine and AMP present in the bath 20 min later were determined by HPLC. Inclusion of 10 μ M norepinephrine was found to decrease adenosine levels by 50% (Figure 3B). Levels of AMP increased by 41%; although

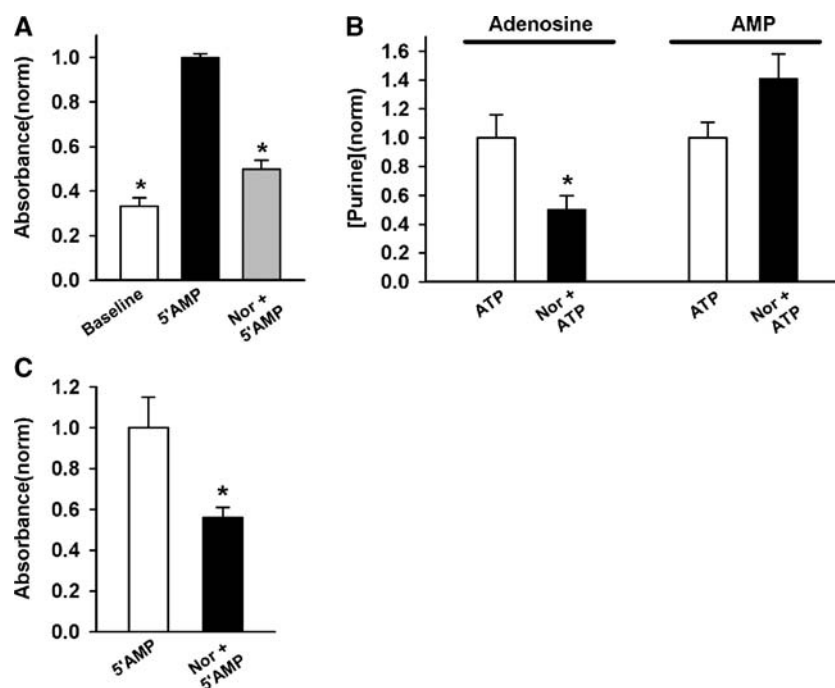


Figure 3. Norepinephrine inhibits ecto-5' nucleotidase activity of RPE cells. (A) Norepinephrine (Nor + 5'AMP, 10 and 100 μ M, respectively) reduced conversion of 5'AMP to Pi. Baseline values were obtained without added 5'AMP. * p < 0.0001 vs. 5'AMP alone, n = 12–13. (B) When 100 μ M ATP was added to the bath surrounding ARPE-19 cells, 10 μ M norepinephrine reduced the amount of adenosine present 20 min later as determined with HPLC analysis (* p = 0.039). The levels of AMP in the bath showed a small but insignificant rise, consistent with an inhibition of ecto-5' nucleotidase (p = 0.095); n = 4. (C) Levels of Pi produced from 100 μ M 5'AMP by the apical membrane of the fresh bovine RPE eyecup were also reduced by 10 μ M norepinephrine (Nor + 5'AMP) * p = 0.017, n = 8–9.

this increase was not significant, the parallel decrease in adenosine and increase in 5'AMP levels is consistent with a decline in catalyzing the dephosphorylation of AMP into adenosine by ecto-5' nucleotidase.

Regulation of activity on fresh bovine RPE by norepinephrine

While the experiments above provide strong evidence that catecholamines down-regulate ecto-5' nucleotidase activity on ARPE-19 cells, the regulation of the enzyme on fresh RPE cells was investigated. As mentioned, the fresh bovine RPE eyecup preparation allows activity to be measured specifically from the apical membrane of RPE cells, providing potential information about adenosine production in sub-retinal space. Experiments were repeated in the fresh bovine RPE eyecup. The production of phosphate was reduced 44% by norepinephrine (Figure 3C). This suggests that stimulation of adrenergic receptors on the apical membrane of fresh RPE can decrease activity of ecto-5' nucleotidase on the same membrane.

Decrease in activity mediated by $\alpha 1$ -adrenergic receptor

The receptor responsible for the effects of norepinephrine on ecto-5' nucleotidase activity was determined pharmacologically. Epinephrine reduced phosphate production from 5'AMP, reducing levels by $87 \pm 3\%$ over baseline ($n = 6$, Figure 4A). The $\alpha 1$ -adrenergic agonist phenylephrine also reduced the production of phosphate; the inhibition was dose-dependent over the range tested (Figure 4B). To confirm a role for the $\alpha 1$ receptor the effect of two different antagonists were examined. At 10 μ M, prazosin completely prevented the effects of norepinephrine (Figure 4C). The antagonist corynanthine also produced a significant inhibition, although 10 μ M of this antagonist led to a partial inhibition (Figure 4D). To rule out contributions from β -adrenergic receptors linked to G_s , the effect of raising intracellular cAMP with a cocktail was examined. The cocktail consisted of 10 μ M forskolin, 100 μ M 3-isobutyl-1-methylxanthine (IBMX) and 500 μ M 8-(4-chlorophenylthio) (cpt) cAMP, and has previously used to produce changes in RPE cells indicative of an elevation in intracellular cAMP [7]. However, the cocktail had no effect on phosphate production (Figure 4E).

Mechanism of catecholamine block

Ecto-5' nucleotidase is linked to the membrane by a glycosylphosphatidylinositol (GPI) anchor, and activity is frequently regulated by changing the amount of enzyme present on the membrane surface. As an initial investigation into the mechanisms underlying the drop in enzyme activity with catecholamines, the effects of temperature and time on the reduction of phosphate production were examined.

Norepinephrine led to a very rapid block of ecto-5' nucleotidase activity. Only 1 min after addition of sub-

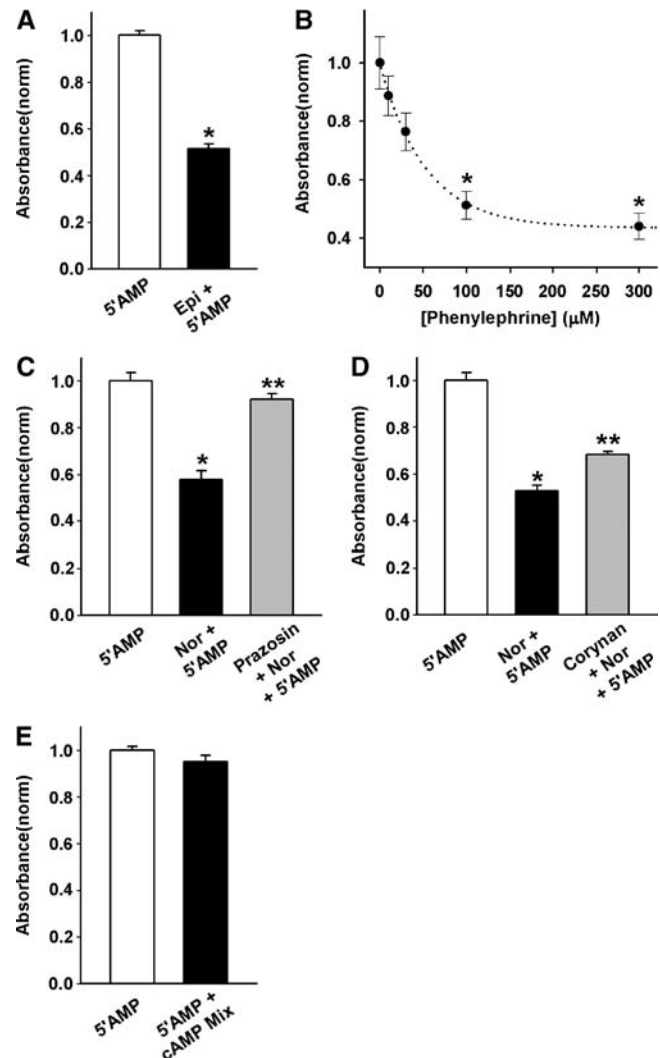


Figure 4. Inhibition of ecto-5' nucleotidase activity mediated by $\alpha 1$ -adrenergic receptor. (A) Epinephrine reduced the 5'AMP-derived production of Pi from ARPE-19 cells. In the presence of 100 μ M 5'AMP (5'AMP), Pi production was inhibited by co-incubation with of 3 μ M epinephrine (5'AMP + Epi) $p < 0.0001$ vs. 5'AMP alone, $n = 6$. (B) The $\alpha 1$ agonist phenylephrine inhibited production of Pi from 5'AMP by ARPE-19 cells in a dose dependent manner. The dotted line is an exponential fit to the data, with $r^2 = 0.99$, $Y_0 = 0.435$, $a = 0.560$ and $b = 0.019$ (see Materials and methods). $*p < 0.05$ vs. 5'AMP alone, $n = 6-11$. (C) The inhibition produced by 10 μ M norepinephrine was prevented by 10 μ M of the $\alpha 1$ adrenergic antagonist prazosin. Levels with prazosin were not significantly different from 5'AMP alone. $*p < 0.001$ vs. 5'AMP, $**p < 0.001$ vs. 5'AMP + norepinephrine, $n = 6$. (D) The $\alpha 1$ adrenergic antagonist corynanthine (corynan, 10 μ M) also blocked the effect of norepinephrine. $*p < 0.001$ vs. 5'AMP, $**p < 0.05$ vs. 5'AMP + norepinephrine, $n = 7-8$. (E) A mixture of 100 μ M IBMX, 10 μ M forskolin and 500 μ M cpt cAMP had no significant effect on phosphate production in the presence of 5'AMP, $n = 9-10$.

strates, phosphate production was reduced by $>60\%$ when compared to the production under baseline conditions (Figure 5A). This reduction was sustained for the 60 min that measurements were made, although the effective block became proportionally less with time. The rapid nature of the block argues against a role for transcription or translation in the decrease. The reduction in phosphate production was not affected by performing the reaction at 4°C as opposed to the normal 37°C (Figure 5B). This implies that changes in

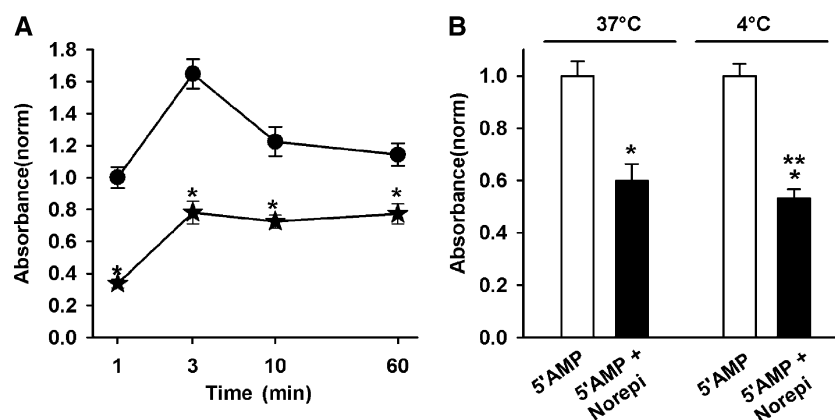


Figure 5. Time and temperature dependence of inhibition. (A) Norepinephrine reduced phosphate production by >60% within the first minute of the reaction, with the reduction remaining throughout 60 min. Circles are 5'AMP alone and stars include 10 μ M norepinephrine. * p < 0.05 vs 5'AMP for a given time point, n = 10–12. (B) The decrease in activity associated with 10 μ M norepinephrine detected at the usual reaction temperature of 37°C was not affected by performing the reaction at 4°C. * p < 0.05 vs. 5'AMP alone, **not significantly different from Nor at 37°C, n = 14–16.

the rate of enzyme internalization or trafficking are not responsible for the decreased activity.

Discussion

The present study has functionally identified the enzyme ecto-5' nucleotidase on RPE cells. Enzyme activity was localized to the apical membrane of bovine RPE cells, the antigen was detected on fresh rat RPE cells and ARPE-19 cultured human RPE cells, while mRNA message and protein for the enzyme was found in ARPE-19 cells. The production of phosphate by ARPE-19 cells was greatly enhanced in the presence of 5'AMP, and this production was prevented by α β mADP. Phosphate production by ARPE-19 cells was also inhibited by epinephrine, norepinephrine and phenylephrine. Norepinephrine decreased the amount of adenosine bathing ARPE-19 cells, and reduced the production of phosphate from 5'AMP by the apical membrane of bovine RPE cells. The effect of norepinephrine on phosphate production from 5'AMP was evident within 1 min and was prevented by α 1 adrenergic antagonists, but reducing the temperature did not alter the block. Together these findings suggest ecto-5' nucleotidase on the apical membrane of RPE cells produces adenosine, and this activity is reduced by stimulating α 1 adrenergic receptors.

Enzyme identification

Several lines of evidence support our conclusion that ecto-5' nucleotidase is the enzyme responsible for the production of phosphate and adenosine on the apical membrane of RPE cells. RT-PCR experiments indicate mRNA message for ecto-5' nucleotidase is present in these cells. The ecto-5' nucleotidase inhibitor α β mADP blocked >50% of adenosine production, and 98% of the phosphate production in the presence of 5'AMP. As α β mADP has no effect on cytoplasmic form of the enzyme [21], this block is consistent with ecto-5' nucleotidase activity. The lack of

phosphate production after incubation with 2'AMP relative to 5'AMP agrees with the substrate preference reported for ecto-5' nucleotidase [22]. A study on airway epithelial cells indicated that both ecto-5' nucleotidase and non-specific alkaline phosphatase were responsible for the degradation of 5'AMP, with alkaline phosphatase contributing 3 fold more at a substrate concentration of 100 μ M [10]. However the alkaline phosphatase inhibitor levamisole did not alter phosphate production from 5'AMP in RPE cells. This is consistent with large block of phosphate production by α β mADP, and with recent data indicating levamisole did not alter the rate of ATP degradation in these cells [13]. This suggests the contribution of alkaline phosphatases to nucleotide degradation in ARPE-19 cells is minimal.

These findings support previous studies demonstrating that the outer retina contains a considerable amount of 5' nucleotidase activity. Close inspection of the lead precipitate found by Kreuzberg and Hussain on albino mice shows the darkest staining at the proximal end of the outer segments [23]. While this staining was interpreted as evidence for activity on Müller cells, the pattern is consistent with activity on the apical membrane of the RPE. An immunohistochemical study of adult albino rat retina also displayed considerable fluorescence at the RPE/outer segment region, although staining at the Müller cells microvilli was more intense, particularly at the tips [24]. This difference may represent a distinction between maximal enzyme levels and maximal activity levels in the retina. However, all reports do stress the importance of the enzyme in the outer retinal region, and emphasize the potential for both P2 and P1 receptors in signaling throughout the outer retina.

Mechanism for ecto-5' nucleotidase regulation

Ecto-5' nucleotidase is linked to the plasma membrane by a GPI anchor and activity is typically regulated by modifying enzyme expression on the membrane. Intracellular pools of enzyme are cycled to the cell surface and either endocytosed to reenter the cycle or cleaved off the anchor [25].

Modulation of enzyme activity has been linked to changes in surface expression, with lovastatin reducing the rate of endocytosis by interfering with Rho-GTPases, and hypoxia reducing endocytosis by increasing the proportion of saturated fatty acids in the membrane [26, 27]. However, endocytosis is temperature dependent; as lowering the temperature from 37 to 4°C had no effect on ability of norepinephrine to decrease activity in the present study, this decrease is unlikely to result from a change in enzyme trafficking. The speed of the change in RPE cell activity shown above also makes transcriptional or translational modulation unlikely to play a role. Adenosine increases activity of ecto-5' nucleotidase through enhanced transcription, but these changes take place over many hours [28]. Likewise, elevation of cAMP increased the synthesis of ecto-5' nucleotidase, but at least 12 h was necessary to detect any increase [29], consistent with our inability to detect an effect of the cAMP cocktail over 30 min.

In the heart, stimulation of the α 1-adrenergic receptor enhances activity of ecto-5' nucleotidase by activating PKC [30, 31]. While this is the opposite of what we have found in the present study, this likely reflects a real difference as we observed this decline with three different agonists, blocked it with two antagonists, corroborated it with changes in adenosine levels and found this decrease in both cultured human and fresh bovine RPE cells. This discrepancy may be explained by a difference in mechanism. The enhancement of activity in cardiac cells took at least 15–30 min after presentation of norepinephrine, and is hypothesized to involve phosphorylation of ecto-5' nucleotidase from inside the membrane. The rapid time scale of our changes suggest the enzyme is instead sheared off the membrane of RPE cells. Stimulation of the α 1 adrenergic receptor can activate phospholipase C (PLC) and activation of phospholipase C can cleave ecto-5' nucleotidase from the membrane [32]. While the activity of the cleaved enzyme generally increases, this increase is dependent upon the lipid constituents of the membrane [32], and a decrease in ecto-5' nucleotidase activity was found after cleavage from endothelial cells by TNF- α [33]. As the space between the apical membrane of the RPE and the photoreceptor outer segments is filled with a complex protein scaffolding [34], ecto-5' nucleotidase cleaved off the RPE cells could subsequently bind to a site on this interphotoreceptor matrix and modulate signaling closer to the outer segments. While our investigations do not enable us to precisely define the mechanism responsible for the decrease on RPE cells, they do emphasize that a particular receptor may have multiple effects on ecto-5' nucleotidase activity, reflecting the many modes for enzyme regulation that are now apparent.

Physiologic implications

The inhibition of ecto-5' nucleotidase activity following stimulation of the α 1 adrenergic receptor links observations by several investigators. Epinephrine is released from the neural retina during the light [15]. Our findings here indicate that epinephrine can reduce ecto-5' nucleotidase

activity, potentially leading to a reduction in adenosine production at light onset. Rod outer segments are shed by the photoreceptors and phagocytosed by the RPE, with peak activity levels at the onset of light [35]. The process of disc shedding and phagocytosis is complex, and the predominant temporal regulator is the circadian shedding of tips. However the rate of phagocytosis itself can be altered, and stimulation of A₂ adenosine receptors in the RPE membrane can decrease this rate [4]. The light-dependent release of epinephrine would lead to less ecto-5' nucleotidase activity and thus less adenosine available to stimulate these receptors, enabling an increase in phagocytosis shortly after light onset. This is supported by studies on pyrimidine 5' nucleotidase where enzyme activity declined after light onset [20]. Although the enzyme preferentially used pyrimidines as a substrate under acid conditions, 5'AMP was used as a substrate at physiologic pH [36], suggesting the regulated enzyme activity was ecto-5' nucleotidase. Our findings are consistent with epinephrine causing this decrease in enzyme activity when lights go on and imply that the activity of ecto-5' nucleotidase may modulate the neurochemical communication between the retinal and RPE.

In addition to altering the light-dependent interactions of the RPE and photoreceptors, the extracellular production of adenosine may have implications for the ability of ATP to trigger signaling in RPE cells. Recently it was suggested that ATP and adenosine act synergistically to raise intracellular Ca²⁺ levels in RPE cells, enabling low levels of ATP to trigger cellular responses [37]. As the elevation of Ca²⁺ by ATP in these cells can lead to enhanced fluid movement [2], this synergism may be key component to RPE physiology. It remains to be seen whether epinephrine can reduce Ca²⁺ signaling by reducing the availability of adenosine available for this synergism.

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